Molecular markers for colorectal cancer screening and prediction of response to therapy

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Chapter 7

Promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing targets the *deleted in colon cancer* (*DCC*) gene in colorectal carcinogenesis without affecting neighboring genes on chromosomal region 18q21

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Abstract

Chromosomal loss of 18q21 is a frequent event in colorectal cancer (CRC) development, suggesting that this region harbors tumor suppressor genes (TSGs). Several candidate TSGs, among which Methyl-CpG-Binding Domain Protein 1 (MBD1), CpG-Binding Protein (CXXC1), Sma- and Mad related protein 4 (SMAD4), Deleted in Colon Cancer (DCC), and Methyl-CpG-Binding Domain Protein 2 (MBD2) are closely linked on a 4-MB DNA region on chromosome18q21. As TSGs can be epigenetically silenced, this study investigates whether MBD1, CXXC1, SMAD4, DCC and MBD2 are subject to epigenetic silencing in CRC. Methylation specific PCR and sodium bisulfite sequencing of these genes show that DCC, but not MBD1, CXXC1, SMAD4 and MBD2, has promoter CpG island methylation in CRC cell lines and tissues (normal mucosa (29.5% (18/61), adenomas (81.0% (47/58)) and carcinomas (82.7% (62/75))($P=8.6 \times 10^{-9}$)) which is associated with reduced DCC expression, independent of 18q21 loss analyzed by multiplex ligation-dependent probe amplification. Reduced gene expression of CXXC1, SMAD4 and MBD2 correlates with 18q21 loss in CRC cell lines (P=0.04, 0.02 and 0.02 respectively). Treatment with the demethylating agent 5-aza-2'-deoxycytidine, but not with the histone deacetylase inhibitor Trichostatin A exclusively restored DCC expression in CRC cell lines. Chromatin immunoprecipitation studies reveal that the DCC promoter is marked with repressive histone-tail marks H3K9me3 and H3K27me3, whereas activity related H3K4me3 was absent. Only active epigenetic marks were detected for MBD1, CXXC1, SMAD4 and MBD2.

This study demonstrates specific epigenetic silencing of DCC in CRC as a focal process not affecting neighboring genes on chromosomal region 18q21.

Introduction

Colorectal cancer (CRC) development is characterized by the growth of a benign precursor lesion from which a small percentage will progress into a carcinoma. Genetic alterations underlying the adenoma to carcinoma transition have been extensively studied over the past two decades. Chromosomal loss of 18q has been reported to occur in 60-70% of CRCs, suggesting this region harbors tumor suppressor genes (TSGs) involved in colorectal carcinogenesis[1-3]. Although chromosomal loss of 18q often affects a large section of the chromosomal arm, a minimal loss region on 18q21, including *Deleted in Colon Cancer* (*DCC*) has been identified[2,4]. Near to *DCC*, within the same 4-Mb chromosomal region, lie several other candidate TSGs, including *Sma- and Mad related protein 4* (*SMAD4*)[5], *Methyl-CpG-Binding Domain Protein* 1 (*MBD1*), CpG-Binding Protein *CXXC1* and *Methyl-CpG-Binding Domain Protein 2* (*MBD2*)[6,7].

Since loss of function for TSGs requires biallelic inactivation, these genes have been examined for the presence of mutations in the remaining allele. For *DCC*[8], *MBD1*, *CXXC1* and *MBD2*[6] mutations are relatively rare events (<5%) in CRC. *SMAD4* has some reports of mutations (15%)[9], which may be higher in tumors with distant metastasis (35%)[10], but even this mutational rate does not match the frequency of loss (60-70%) at 18q21. While haplo-insufficiency might provide another explanation for the observed low mutation frequencies[11], this raises the question of whether alternative mechanisms might account for inactivation of these TSGs.

In addition to genetic changes in cancer, epigenetic modifications including promoter CpG island methylation are associated with gene silencing and serve as mechanism to inactivate tumor suppressor- and DNA repair genes[12]. Promoter CpG island methylation is often associated with histone modifications[13]. Histone 3 trimethyl lysine 9 (H3K9me3) or lysine 27 (H3K27me3) marks are associated with methylated DNA and transcriptional silencing[14]. In contrast, acetylation of histone H3[15] and trimethylation of histone H3 at lysine 4 (H3K4me3) is associated with unmethylated DNA and gene expression and can be considered as marks of active chromatin[13].

While chromosomal region 18q21 deletion occurs frequently in colorectal carcinogenesis and has been extensively studied for genetic mechanisms potentially leading to gene inactivation[16], little is known about epigenetic silencing of genes in this region. Therefore, in this study we investigate whether epigenetic mechanisms serve as second hit in inactivating genes on chromosome 18q21 in colorectal cancer.

Materials and Methods

Cell lines, study population and tissues

CRC cell lines (CACO2, COLO205, HT29, SW480, HCT116, RKO and LS174T and SW48) were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone).

MBD1, *CXXC1*, *SMAD4*, *DCC* and *MBD2* promoter CpG island methylation was investigated in a well-characterized series of sporadic colorectal carcinomas. The series consists of formalin-fixed, paraffin-embedded CRC tissues (n=75) of patients over 50 years of

age, 37 males and 38 females (mean age 71.0, range 50-88, at time of diagnosis) which were retrospectively collected from the tissue archive of the dept. of Pathology of the Maastricht University Medical Center. When present, also normal (n=61) and adenoma (n=58) tissue was collected from these patients. Histological normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n=48) were selected (23 males, 25 females, age 63.9, range 50-87 years at time of diagnosis). Clinicopathologic characteristic are listed in Supplementary Table 1. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University Medical Center.

DNA isolation and bisulfite treatment

A 5µm section of each tissue block was stained with haematoxylin and eosin andreviewed by a pathologist (AdB). Five sections of 20µm were deparaffinated prior to DNA-isolation. DNA was extracted using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturers instructions. Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co, Orange, CA) according to the manufacturers instructions.

Promoter CpG island methylation analyses: sodium bisulfite sequencing and methylation specific PCR

For sequencing of sodium bisulfite-converted DNA, PCR products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands). Single colonies were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA).

MSP analysis on bisulfite treated DNA were performed as described in detail elsewhere[17,18]. To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue, DNA was first amplified with flanking PCR primers which was used as a template for the MSP reaction. All primer sequences and conditions are shown in Supplementary Table 2.

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was performed as described before[19,20]. In brief, an oligonucleotide MLPA probe set was designed for 45 genes on chromosomes 3, 5, 8p, 9, 11q,15q,18q, and 19q. This mix contained probes for eight genes located on chromosome 18q, including probes for *DCC* and *SMAD4*. Additionally, eleven reference probes (two probes on chromosome 2p; three probes on chromosome 4q; two probes on chromosome 12; and four probes on chromosome16) were included for quality control and normalization purposes. Data analysis was performed using excel-based software developed at MRC-Holland, which provides a reliable and robust normalization for MLPA fragment data files[21]. The data was normalized using the median of the reference probes. As reference DNA, a pool of DNA extracted from normal paraffin embedded tissue samples of different organs (10 colons, 10 stomach, 4 kidneys, 4 liver and 4 spleen) was used. DNA copy number ratios were obtained by dividing the median area under the peak for each probe in the sample by the median area under the peak of at least three reference DNA samples. Each sample was run at least three times, and the median of the different runs were used for analysis.

Finally, all DNA copy number ratios were normalized by setting the median copy number ratio of the reference probes to 1.0. A ratio less than 0.8 was considered as a deletion, and a ratio higher than 1.2 as a gain.

Quantitative reverse transcriptase PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturers instructions. Possible genomic DNA contaminations were removed by DNAse treatment with the RNase-free DNAse set (Qiagen). cDNA synthesis was performed using the Iscript cDNA synthesis kit (Bio-Rad). Quantitative real-time RTPCR was performed as described previously[22] using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). *MBD1*, *CXXC1, SMAD4, DCC* and *MBD2* expression levels were normalized to *CYCLOPHILIN* and average expression levels in normal colon- adjacent to carcinoma tissues (n=10) for each tissue sample using the following equation: relative expression = $2^{-(\Delta Ct)}$, where ΔCt = average Ct (tissue sample) - average Ct (CYCLOPHILIN) - average Ct (normal colon tissues). Since alterative splicing of *DCC* exons 6-7 and 18-23 has been reported[23,24], *DCC* RT-primers are located in exon 2-3.

To investigate re-expression of *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* following inhibition of DNA methyltransferases or inhibition of histone deacetylation, HCT116, RKO cells and HT29 cells were treated with 5 µM 5-aza-2'-deoxycytidine (Aza) (Sigma) and with 300 nM Trichostatin A (Sigma) for 96 and 18 hours respectively.

As a positive control for gene expression following inhibition of DNA methyltransferases re-expression of *GATA4* and *GATA5* were used. As a positive control for gene expression following treatment with Trichostatin A (TSA), the previously described control gene *FABP4*[15,25] was analyzed.

Chromatin immunoprecipitation (ChIP) assays

ChIPs were performed for cell lines HCT116 and HT29 cells and analyzed essentially as described previously [26]. Briefly, HCT116 and HT29 cells were fixed in 1% formaldehyde. Samples were immunoprecipitated with antibodies for either HA as a negative control (sc-805; Santa Cruz), H3K9me3 (07-442;Upstate), H3K27me3 (07-449; Upstate) and H3K4me3 (ab8580; Abcam). Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as negative control and subtracted from enrichment values. For *MBD1*, *CXXC1*, *SMAD4* and *MBD2,* three primer pairs were designed covering -600 bp to the TSS. For *DCC,* six additional primer pairs were designed to investigate the region -597bp to +467 bp relative to the TSS. Control PCRs for each antibody immunoprecipitation were performed using primers for *GAPDH* and *P16^{INK4A}* (monoallelic methylation in HCT116 and biallelic methylation in HT29 (data not shown)) as negative and positive control dependent on the antibody used.

Data analysis

We used the Pearson's χ2 or Fisher's Exact test, students t-test, Kruskal-Wallis, Mann-Witney or Wilcoxon rank test where appropriate to compare categorical and continuous data, respectively. Since a significant difference in age was observed between CRC cases and controls (P= 1.1 x 10^{-4}), logistic regression analyses were used to adjust for age. All

quoted P values are two-sided, and a *P* value < 0.05 was considered statistically significant. Where appropriate, the Bonferroni method was used to correct for multiple comparisons. Data analysis was done using SPSS software (version 12.0.1).

Results

DCC promoter is methylated in colorectal cancer cell lines, while MBD1, CXXC1, SMAD4 and MBD2 are unmethylated

We first analyzed promoter CpG island methylation of 5 TSGs genes within a 4-Mb region in proximity to *DCC* (46.0 Mb to 49.9 Mb) including *MBD1*, *CXXC1*, *SMAD4, DCC* and *MBD2.* Using public ENSEMBL and EMBOSS CpG plot/CpG software we observed that *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* each have a CpG island-associated promoter region (GC content > 60%, ratio of observed CpG / expected CpG > 0.6 and minimum length 200 bp[27]). For an initial assessment of the methylation status of these promoter CpG islands methylation specific PCR (MSP) was performed on colorectal cancer (CRC) cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Since *DCC* has 2 CpG rich regions, region 1 located -95 bp to +170 bp and region 2 located +608 bp and +962 bp relative to the transcription start site (TSS), both regions were analyzed. *MBD1*, *CXXC1, SMAD4* and *MBD2* promoter CpG island methylation status was analyzed using primers for the CpG islands spanning the TSS (primers, locations and PCR conditions are provided in Supplementary Table 2). All CRC cell lines have complete (CACO2, COLO205, HT29, SW480, HCT116, RKO, SW48) or partial (LS174T) *DCC* DNA methylation in region 1, while region 2 showed complete *DCC* methylation for all cell lines. In contrast, CpG-rich regions within *MBD1*, *CXXC1, SMAD4* and *MBD2* promoters were unmethylated in all cell lines analyzed (data not shown).

To study in more detail the DNA methylation of these promoter regions, sodium bisulfite sequencing of individual clones of CRC cell lines HCT116 and RKO and 3 paired normal and adjacent CRC tissues showed that both *DCC* CpG islands are densely hypermethylated in CRC tissues while normal tissues showed less methylated CpG dinucleotides (Figure 1). This difference is most apparent for the promoter region of *DCC* (region 1), in which normal tissues 1, 4 and 8 have, respectively, 7.3%, 1% and 3.1% of sequenced CpG dinucleotides methylated, while in the more 3' region (region 2), 40.3%, 22.2% and 25% of sequenced CpG dinucleotides were methylated. Since the promoter region (region 1) demonstrated the most cancer specific methylation and epigenetic changes are most tightly associated with transcription near the transcriptional start site, we continued this analysis for *DCC* in region 1.

In contrast to *DCC*, the *MBD1*, *CXXC1*, *SMAD4* and *MBD2* gene promoter regions are almost completely unmethylated at all CpG dinucleotides.

Reduced CXXC1, SMAD4 and MBD2 gene expression associates with 18q21 loss

To determine the effect of promoter CpG island methylation and loss of 18q21 on gene expression, we analyzed DNA copy number changes of 8 loci on region 18q harboring *SMAD4* (46.8 Mb) and *DCC* (48.1 Mb) in detail by multiplex ligation dependent probe amplification (MLPA)[28] in CRC cell lines (Figure 2A). These analyses revealed that cell lines

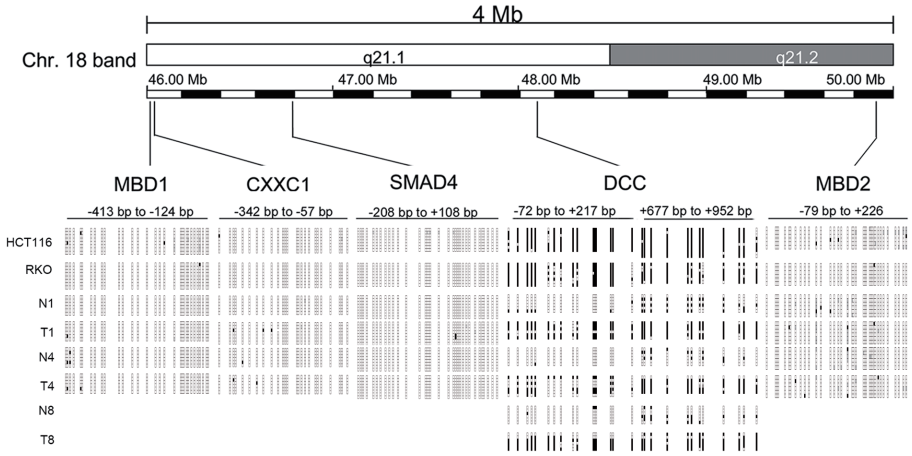

Figure 1. Sodium bisulfite sequencing results of MBD1, CXCC1, SMAD4, DCC and MBD2 gene promoters on cell lines HCT116 and RKO and normal and adjacent tumor tissues of three patients (1, 4 and 8) show that the DCC promoter is densely methylated. Each row represents an individual cloned allele and each square indicates a CpG dinucleotide. Black square, methylated CpG site; white square, unmethylated CpG site; gray square, not determined.

CACO2, COLO205, HT29 and SW480 had loss of the complete region tested, including the *DCC* locus. This was not observed for cell lines HCT116, RKO, LS174T and SW48 which had no loss of 18q21.

Next we studied *MBD1, CXXC1*, *SMAD4, DCC* and *MBD2* gene expression and the relation to 18q21 deletion in CRC cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Expression of *CXXC1*, *SMAD4* and *MBD2* was significantly higher in cell lines HCT116, RKO, LS147T and SW48 in which 18q21 was retained, compared to CACO2, COLO205, HT29 and SW480 cells in which 18q21 is deleted (Mann-Whitney, *P*=0.04, 0.02 and 0.02 respectively) (Figure 2B). *MBD1* expression was detectable in all CRC cell lines but did not correlate to 18q21 loss. In contrast, *DCC* mRNA levels were nearly undetectable in all CRC cell lines, while clearly present in positive control brain tissue[23] and normal colon tissue, suggesting that complete DNA methylation observed in these cell lines correlated with absent expression.

DCC promoter methylation is a frequent and early event in colorectal carcinogenesis

Next, promoter CpG island methylation of these 18q21 genes was studied in a well characterized series of formalin-fixed, paraffin-embedded primary CRCs (n=75). Where available, adenoma- (n=58) and normal tissue (n=61) of the same patients was analyzed also. This analysis showed that *DCC* promoter methylation is a common event in CRC, which occurred in 82.7% (62/75) of CRCs, 81% (47/58) of adenomas and 29.5 % (16/61) of normal colon mucosa of CRC patients (P= 8.6 x 10^{-9}) (Table 1).

For 50 patients normal, adenoma and carcinoma tissue was available allowing a detailed analysis of *DCC* promoter methylation in colorectal carcinogenesis (Table 2). These analyses showed that for 13 patients, carcinoma-, adenoma- and normal tissues were synchronously methylated indicating that *DCC* promoter methylation occurs early in CRC development. *DCC* DNA promoter methylation was also studied in age-matched

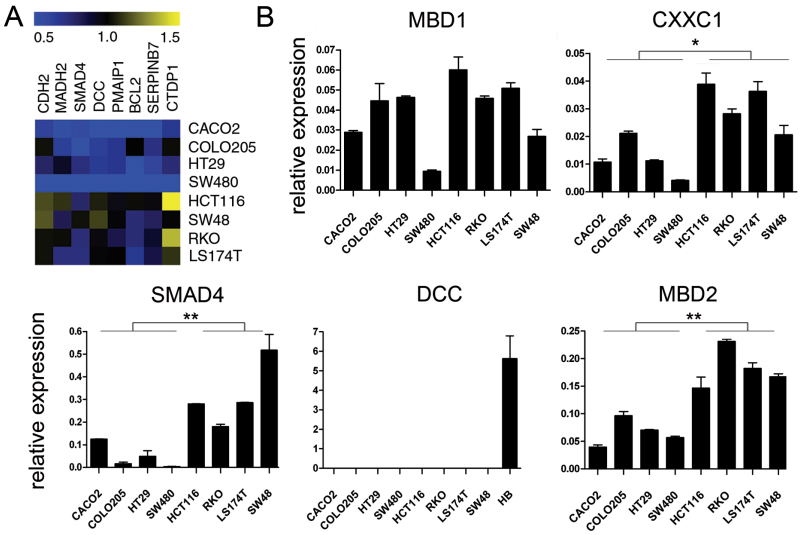

Figure 2. (A) Heatmap representation of MLPA that shows loss of eight loci on chromosomal region 18q in cell lines CACO2, COLO205, HT29 and SW480. A ratio <0.8 (indicated by blue) was considered as a deletion, a ratio >1.2 (indicated by yellow) as a gain. **(B)** Quantitative reverse transcription–PCR of *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* on CRC cell lines. Gene expression levels are relative to the mean expression level of the normal colon tissues (n=10), which is set to equal 1. Figure shows that *DCC* transcript levels are nearly undetectable in all CRC cell lines, whereas high expression was present in human brain (HB). Expression of *MBD1*, *CXXC1*, *SMAD4* and *MBD2* is lower in CRC cell lines when compared with normal colon- adjacent to carcinoma tissues (n=10). Expression of *CXXC1*, *SMAD4* and *MBD2* was significantly lower in cell lines with 18q21 deletion when compared with cell lines without 18q21 deletion. Data represent mean and standard error of mean of two or three independent experiments. Mann–Whitney, **P*=0.04. Mann–Whitney ***P*=0.02.

normal colorectal mucosa tissues of 48 non-cancer patients of which 20.8% (10/48) was methylated.

MBD1, *CXXC1, SMAD4* and *MBD2* gene promoters were unmethylated in the vast majority of CRCs (32/33, 27/32, 28/29,31/32 respectively), adenoma (32/33, 33/34, 30/32,31/32 respectively) and normal tissues (31/31, 32/32, 32/32, 31/31 respectively). (Table 1). Remarkably, in 1 carcinoma *CXXC1, SMAD4, DCC* and *MBD2* were synchronously methylated.

18q21 copy number change complements DCC promoter methylation

Table 1. Promoter methylation analyses (MSP) on normal- and CRC tissues

tissue	*MBD1*	*CXXC1*	*SMAD4*	*DCC*	*MBD2*
Normal colon				10/48 (20.8%)	
CRC tissues					
normal	0/31	0/32	0/32	16/61 (29.5%)	0/31
adenoma	1/31	1/34	2/32	47/58 (81%)	1/32
carcinoma	1/31	5/32	1/29	62/75 (82.7) *	1/32

* *P*=8.6 x 10-9

Next we studied how 18q21 deletion and DNA promoter methylation collaborate in transcriptional gene silencing in a second series of normal and adjacent CRC tissues of patients (n=20) of which frozen tissue was available. In this series *DCC* promoter methylation was detected in 75% (15/20) of CRCs (Figure 3A). In the paired normal tissues, only 5% (1/20) was methylated. *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters were all unmethylated in these tumors (data not shown). Deletion of 18q21 was detected in 40% (8/20) of cancer tissues and in none of the normal tissues. In all detectable 18q21 deletions the *DCC* locus was included and in 7 of these 8 CRCs all probes on 18q showed DNA copy number loss. Thereby, we show that *DCC* is affected by promoter CpG island methylation and/or loss of the *DCC* locus in 90% of CRCs. No specific correlation between DNA promoter methylation and 18q21 copy number loss was observed.

The effect of *DCC* promoter methylation and 18q21 loss on gene expression was studied in 10 of the 20 patient samples for which RNA was available (Figure 3B). In this subset *DCC* promoter methylation was observed for all CRCs, and 1 normal tissue (patients 16). Although inter-individual differences in gene expression levels were observed, quantitative real-time analyses showed that *DCC* expression was significantly reduced in all CRCs when compared to matching normal tissues. Very low *DCC* transcript levels were detected in both normal and carcinoma tissue of patient 16 which corresponds to the methylated status of both tissues. Apart from tumor T11, in which *DCC* expression was more selectively targeted, most of the carcinoma tissues showed reduced expression of *MBD1*, *CXXC1*, *SMAD4* and *MBD2* as well. In T20, characterized by loss of 18q21, a strong reduction in expression of all genes was observed. However, in contrast to the correlations observed in cell lines, reduced gene expression of *CXXC1*, *SMAD4* and *MBD2* was not restricted to tumors with loss of 18q21, indicating the presence of other transcriptional silencing mechanisms for *MBD1*, *CXXC1*, *SMAD4* and *MBD2* in CRC.

Table 2. *DCC* promoter methylation in CRC patients

Patient	normal	adenoma	carcinoma
1	■	■	■
2	□	■	■
3	■	□	□
4	□	■	□
5	□	□	■
6	□	□	■
7	□	■	■
8	□	■	■
9	■	□	■
10	□	■	□
11	□	■	■
12	■	■	■
13	■	■	□
14	□	□	■
15	■	■	■
16	□	■	■
17	□	■	■
18	□	□	■
19	■	■	□
20	□	□	□
21	□	■	■
22	□	■	■
23	□	■	■
24	□	■	■
25	□	■	■
26	□	□	■
27	□	■	■
28	■	■	■
29	■	■	■
30	□	■	■
31	■	■	■
32	■	■	■
33	□	■	■
34	■	■	■
35	□	□	□
36	□	■	■
37	□	■	■
38	■	■	■
39	■	■	■
40	□	■	■
41	■	■	■
42	□	■	■
43	□	■	■
44	□	■	■
45	□	■	□
46	□	■	■
47	■	■	■
48	□	■	■
49	□	■	■
50	■	■	■

DCC promoter CpG island methylation analysis by MSP on matched normal-, adenoma- and carcinoma tissues of 50 patients. Grey- and white boxes represent methylated- and unmethylated genes, respectively.

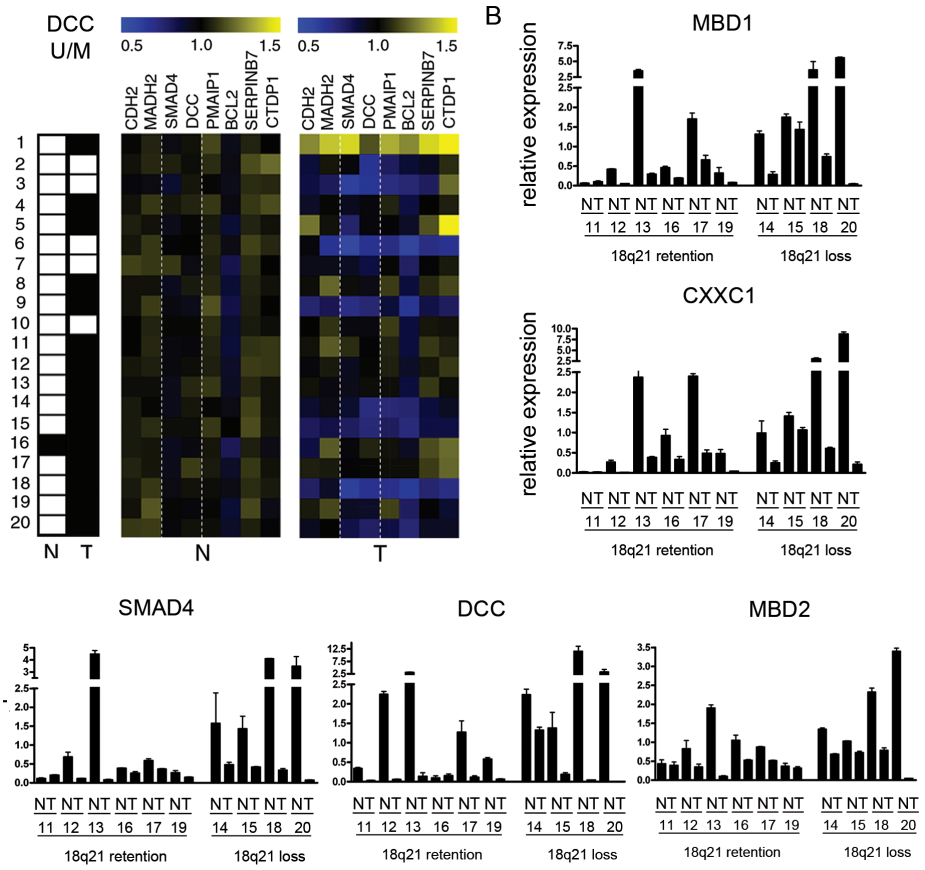

Figure 3. Integrative analysis of DCC DNA promoter methylation and chromosomal loss of region 18q21 and their relation to transcriptional gene silencing in CRC tissues.
(A) Heatmap representation of MLPA that shows loss of eight loci on chromosomal region 18q (A) and their relation to DCC promoter methylation. Black and white boxes represent methylated (M)- and unmethylated (U) genes, respectively. **(B)** Quantitative reverse transcription–PCR of MBD1, CXXC1, SMAD4, DCC and MBD2 on paired normal and carcinoma tissues of 10 patients. Figure shows gene expression levels of normal and carcinoma tissues relative to the mean expression level of the normal colon- adjacent to carcinoma tissues together, which is set to equal 1.

Demethylating agents, but not HDAC inhibitors, restore DCC expression

In order to directly investigate the roles of DNA promoter methylation or histone deacetylation in transcriptional silencing of 18q21 genes, HCT116, RKO and HT29 cells were treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza) and the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). Aza restored *DCC* expression in HCT116, RKO and HT29 cells (Figure 4A). Of the unmethylated genes, *MBD1* showed a modest increase in expression after treatment with Aza, while no change in expression was observed for *CXXC1*, *SMAD4 and MBD2* (Figure 4B). TSA was unable to reactivate *DCC* or increase expression of *MBD1, CXXC1*, *SMAD4 and MBD2.* These results indicate that promoter CpG island methylation, but not histone deacetylation

alone, accounts for transcriptional silencing of *DCC*. *MBD1, CXXC1*, *SMAD4 and MBD2* expression is not dependent upon these processes.

DCC promoter CpG island methylation is associated with inactive chromatin marks

We then analyzed the presence of inactive (H3K9me3 and H3K27me3) and active (H3K4me3) chromatin marks on *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* promoter regions with chromatin immunoprecipitation (ChIP) assay for cell line HCT116 (18q21 retention) and cell line HT29 (18q21 copy number loss). ChIP in HCT116 demonstrated that the entire *DCC* promoter and adjacent 3' region have enrichment of the repressive histone-tail marks H3K9me3 and H3K27me3, and depletion of the active chromatin mark H3K4me3 (Figure 5A). As a control, HCT116 had enrichment of H3K9me3 and H3K4me3 but not H3K27me3 on the *P16^{INK4A}* promoter which is consistent with a recent report by Kondo *et al*[29] and with the known active and silent alleles in this cell line[30]. Conversely, the *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters have enrichment of the active H3K4me3 mark, but not of the repressive histone-tail marks H3K9me3 and H3K27me3. In cell line HT29, a similar pattern was observed (Figure 4B), with active marks for all genes, except *DCC*, and repressive marks at *DCC,* although the pattern was slightly different with enrichment for only H3K27me3 and not H3K9me3 at *DCC*. Thus, ChIP analyses showed a clear distinction for the *DCC* gene, with the *DCC* promoter surrounded by inactive chromatin, while *MBD1*, *CXXC1*, *SMAD4* and *MBD2,* have enrichment for active histone tail-marks and no signs of epigenetic gene silencing.

Discussion

In this study we investigated epigenetic silencing of 5 genes, *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2,* located on a 4 Mb region on chromosome 18q21; a chromosomal region which becomes frequently lost during colorectal carcinogenesis[1]. Although a variety of studies have investigated genetic silencing of genes on this chromosomal region, the contribution of epigenetic modifications is still unclear.

With promoter CpG island methylation analysis we showed for the first time, that the *DCC* promoter is densely methylated in CRC cell lines and primary CRC tissues. *DCC* promoter CpG island methylation has been described in oral squamous cell carcinomas[31], head and neck squamous cell carcinomas[32], breast cancer[33], gastric cancer[34] and esophageal squamous cell carcinoma (ESCC)[35,36]. In CRC, research on inactivation of *DCC* has mainly focused on loss of heterozygosity (LOH) and gene mutation. *DCC* is affected by DNA promoter methylation and/or loss of the *DCC* locus in 90% of CRCs. While 18q21 LOH mainly occurs in advanced lesions[3], *DCC* promoter methylation occurs early in CRC development, being present in 80% of adenoma and carcinoma tissues and 29% of normal tissues.

To the contrary *MBD1*, *CXXC1*, *SMAD4*, and *MBD2* promoter CpG island methylation is rare in CRC. However, all five 18q21 genes show reduced gene expression in CRC cell lines and primary tissues compared to normal colon. *CXXC1*, *SMAD4* and *MBD2* reduced expression in cell lines was associated with 18q21 copy number loss, but in primary cancer tissues low mRNA levels were not restricted to CRCs with 18q21 deletion, indicating the presence of other targeting mechanisms. However, none of the genes

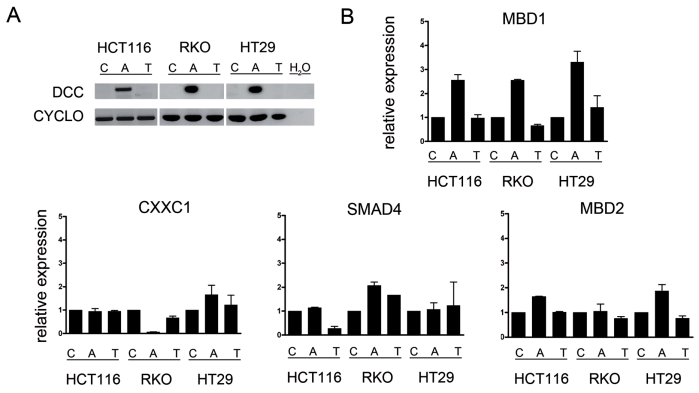

Figure 4. (A) Treatment with 5 lM Aza for 96 h, but not 300 nM TSA for 18 h, restores DCC expression in HCT116, RKO and HT29 cells. C, control; A, treatment with Aza; T, treatment with TSA **(B)** Quantitative reverse transcription–PCR of MBD1, CXXC1, SMAD4 and MBD2 on HCT116, RKO and HT29 cells treated with Aza and TSA, respectively. Gene expression levels in untreated control were set to equal 1. Data represent mean and standard error of mean of two or three independent experiments.

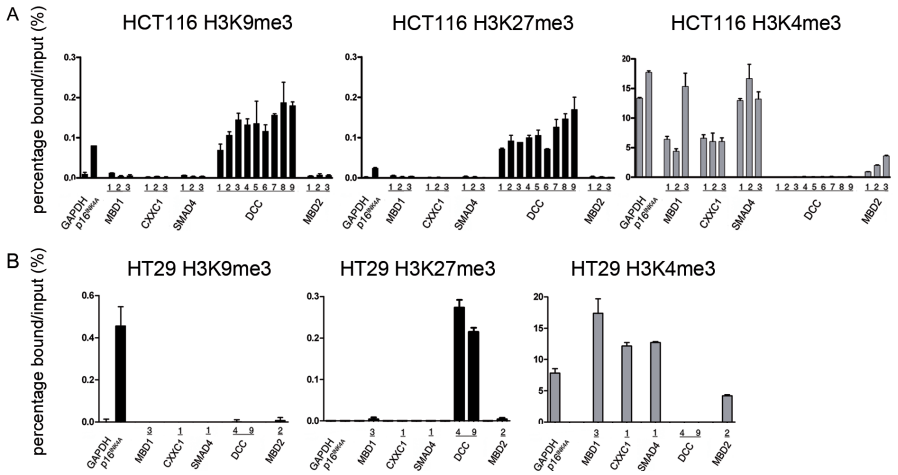

Figure 5. ChIP studies in HCT116 **(A)** and HT29 cells **(B)** reveal that the DCC promoter is enriched with repressive histone-tail marks (H3K9me3 and H3K27me3), whereas MBD1, MBD2, CXXC1 and SMAD4 gene promoters are associated with the active chromatin mark H3K4me3. Immunoprecipitated DNAwas quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as a negative control and subtracted from enrichment values. Control PCRs for each antibody immunoprecipitation were performed using primers for GAPDH and P16INK4A as negative and positive control dependent on the antibody used. Figure shows a representative experiment for two or three ChIPs per antibody.

responded to treatment with histone deacetylase inhibitor Trichostatin A (TSA) and only *DCC* expression was restored by the demethylating agent 5-aza-2'-deoxycytidine (Aza) in CRC cell lines. ChIP studies revealed that only the *DCC* promoter CpG island methylation is associated with repressive histone-tail marks H3K9me3 and H3K27me3 and not with active chromatin mark H3K4me3. The opposite was observed for unmethylated *MBD1, CXXC1*, *SMAD4* and *MBD2* promoters which are enriched with active histone-tail mark H3K4me3 and not with H3K9me3 and H3K27me3. Therefore we conclude that

promoter CpG island methylation and histone modifications specifically target *DCC* on chromosomal region 18q21, while neighboring genes *MBD1, CXXC1*, *SMAD4* and *MBD2* remain unaffected.

In general, epigenetic gene inactivation is considered a gene- and tissue specific process[37]. However, Frigola *et al.* recently showed that epigenetic modifications can cover large chromosomal regions in CRC as well, a mechanism termed long-range epigenetic silencing (LRES)[38]. In this study, genes located on a 4-Mb domain on chromosomal region 2q14 are coordinately epigenetically suppressed by H3K9 methylation, which occurs independently of promoter CpG island methylation. Recently, the same mechanism was observed for chromosomal region 3p22 which flanks the *hMLH1* gene[39] and chromosomal region 5q32.2[40]. Here we show for chromosomal region 18q21 that this type of long-range epigenetic silencing was not observed in cell lines HCT116 (18q21 retention), and HT29 (18q21 loss). Interestingly, in 1 primary CRC *CXXC1, SMAD4, DCC* and *MBD2* were synchronously methylated, however this was not observed for the other tumors (n=31) nor in any colorectal cancer cell line.

The observation that only *DCC* is inactivated by promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing, while *MBD1*, *CXXC1*, *SMAD4* and *MBD2* are not, raises questions about which underlying mechanism leads to this gene specificity. Recent studies have shown that genes which become *de novo* methylated in cancer are often bivalently marked by both active (H3K4me3) and repressive (H3K27me3) histone marks in human embryonal stem cells[14,41]. In adult cancer cells H3K4me3 is diminished and TSGs generate a fully repressive chromatin state[14]. H3K27me3-marked loci are often occupied by Polycomb Group repressive complexes (PRC). Interestingly, in a genome-wide ChIP-ChIP assay which identified target genes of the human Polycomb Group protein SUZ12 in human embryonic stem (ES) cells[42], the *DCC* locus was found enriched for H3K27me3 and SUZ12 in its promoter region, whereas *MBD1*, *CXXC1*, *SMAD4* and *MBD2* were not. As is clear for other cancer associated hypermethylated genes[14], these findings seem to suggest an inherent susceptibility of *DCC* for aberrant methylation during cancer progression.

In summary, this study shows how genetic and epigenetic alterations collaborate in transcriptional silencing of *DCC*. While chromosomal loss of 18q21 frequently involves the entire chromosomal region, promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing specifically targets the *DCC* gene. Long-range epigenetic gene silencing has been observed on chromosomal region 2q14, 3p22 and 5q32.2, but on 18q21 a rather fine-tuning role for epigenetic gene silencing is observed.

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Supplementary Figures and Tables

Supplementary Table 1: Clinicopathologic characteristics of normal, adenoma and carcinoma tissues

	normal colon	CRC patients			*P* value
		normal	adenoma	carcinoma	
age (mean, SD)	63.86 (50-87)	71.2 (51-88)	71.52 (51-88)	71.0 (50-88)	1.1×10^{-4}
sex (%)					
M	47.9	55.7	53.4	49.3	
F	52.1	44.3	46.6	50.7	NS
localisation (%)					
proximal	25	43.1	32.7	47.9	
distal	75	56.9	67.3	52.1	NS
Histological type (%)					
tubular			62.1		
villous			1.7		
tubulo-villous			36.2		
Dysplasia (%)					
low grade			87.9		
high grade			12.1		
Differentiation (%)					
well				12	
moderate				76	
poor				12	
TNM (%)					
I				13.3	
II				32	
III				44	
IV				10.7	

TNM, Tumor, Node, Metastasis; NS, not significant

Supplementary Table 2A: Primer locations, sequences and conditions

Experiment	Gene	Temp	Cycles	type	Location	Primer sequence	
MSP	MBD1	56	35	MSP_Fup/down	-386 to -228	GTATYGGTTGTGGAGAT	CAACTAACCCCTTCRAAAAACTA
		68	35	MSP_U/as		GAGGGAAGGGAGGGTGTAGGTGTAAGTTTTTTTT-GATT	TACAACTCTCCTCACTACCCATCTAACTAACA-CATACA
		68	35	MSP_Ms/as		GAGGGCGTAGGCGTAAGTTTTTTTCGATC	CTCGCTACCCATCTAACTAACGCATACG
	CXXC1	56	35	MSP_Fup/down	-303 to -171	ATGTACGGAATTAATTATTAAGATGG	CATAAATACCRTCATCTTAAAATCC
		62	35	MSP_U/as		GAATTAATTATTAAGATGGTGGTGTTT	TCATCTTAAAATCCAATTCCATACA
		62	35	MSP_Ms/as		ATTATTAAGATGGCGGCGTTC	ATCTTAAAATCCGATTCCGTACG
	SMAD4	56	35	MSP_Fup/down	-296 to +117	GYGTTTTTTGGATATTTTTTTGTAA	TCTAAACCTAAACCTAAACCTCAAC
		66	35	MSP_U/as		TTTGTAATGAGATGTTAATTTTTTTGGT	CCTAAACCTAAACCTCAACACAAACA
		66	35	MSP_Ms/as		TAACGAGATGTTAATTTTTTCGGC	CCTAAACCTCAACGCGAACG
	DCCI	56	35	MSP_Fup/down	+28 to +180	GTGTAGTATGGTTTTAATTTTTAGTTTGTATATT-GTTGGT	AAAACCRAAAAAACCCAAAAA
		68	30	MSP_U/as		TAGTACGGTTTTAATTTTTAGTTCGTATATCGTTGGC	AACCAAAAAAAACCCAAAAAAAAAAAAA-CAAAAAAAACTACA
		68	25	MSP_Ms/as		TATTTTTYGGAGTTTTTTTGTTTAG	CCGAAAAAACCCAAAAAAAAAAAAC-GAAAAAAACTACG
	DCCII	56	35	MSP_Fup/down	+728 to +841	TTTTTTGTTTAGTGTGTATTTTTAAGTAATT	CAAATAAAATAAAAAAAAAAATAAAATAC
		60	35	MSP_U/as		TGTTTAGCGCGTATTTTTAAGTAATC	AAAAAATAAAATACAAAAAAAAATACAACA
		66	35	MSP_Ms/as		TGTTTAGCGCGTATTTTTAAGTAATC	AAATAAAATACGAAAAAAAATACGACG
	MBD2	56	35	MSP_Fup/down	+5 to +187	YGTGGTTTYGAGAAGG	RAACCCTTAAAATCCCRAAACCC
		66	30	MSP_U/as		GAAGGTGGAGATAAGATGGTTGTTTATAGTG	CACAAAACAACCCTCCTACCCAAAACA
		66	30	MSP_Ms/as		CGGAGATAAGATGGTCGTTTATAGC	GACCCTCCTACCCGAAACG
bisulfite sequencing	MBD1	62	40	Bsseq up/down	-413 to -124	GTAGGYGYGAAGTAGTTTTTTTGT	TACCCTTAATAAAAAAACTCCC
	CXXC1	58	40	Bsseq up/down	-342 to -57	TTATTTTATTTGTGAATTTTTTTGTT	CTCTAATATAACTTTCCCRAAAAC
	SMAD4	61	40	Bsseq up/down	-208 to +108	TTGAGGTTTAGGTTTAGGTTTAGAT	AAAAAACAAAAAAAACCTCCC
	DCCI	56	40	Bsseq up/down	-72 to +217	TTTATATTTTTTTTTTTAGTTTTTTATTT	AAAAAAAAATAAAAAACAATCCTATAA
	DCCII	60	40	Bsseq up/down	+677 to +952	GTAAGTGGTTTTTTTTTTTTTTTT	CAAACCAACCCAAATATTTACAA
	MBD2	64	40	Bsseq up/down	-79 to +226	TATGATATTTTTAGTTGGTGGTTAGTT	AACCCCCTCCCCAACC
qRT-PCR	MBD1	60	40	RT_PCR	exon 2-3	GGACGCTCAGACACCTATTACCA	GTTTGAAGTCGAAGAGGGTGAGAT
	CXXC1	60	40	RT_PCR	exon 7-8	CTACAGCCACACCTGAGCCA	TTCTGTGTCGCTCATCCAGG
	SMAD4	60	40	RT_PCR	exon 12-13	GGAAACATCCCTGGCCC	AACTCATCCTGAGTATGCATAAGC
	DCC	60	40	RT_PCR	exon 2-3	GGGACTTTACCAATGTGAGGCA	TTCTGTCTGTGAAAGGAACCTCAGT
	MBD2	60	40	RT_PCR	exon 7-8	AACCCTGCTGTTTGGCTTAAC	CCATCAGTGCTTCTTCCAATTT

Supplementary Table 2B: Primer locations, sequences and conditions

Experiment	Gene	Temp	Cycles	type	Location	Primer sequence	
ChIP	GAPDH	60	40	ChIP	-43 to +52	CGCCCCCGGTTTCTATAAAT	CCTGGCGACGCAAAAGAAG
	$P16^{INK4A}$	60	40	ChIP	-388 to -280	ACCCCGATTCAATTTGGCAG	AAAAAGAAATCCGCCCCCG
	MBD1_1	60	40	ChIP	-484 to -383	GGGACCCACGAACACAGGATA	AACGCGGCAAGGAAGCTG
	MBD1_2	60	40	ChIP	-413 to -289	GCAGGCGCGAAGCAGC	ATGCGCTTTCCAGCCTCAA
	MBD1_3	60	40	ChIP	-74 to +6	AGAGGCAGGCAGCGGACTT	CGGCCGCCTCCTCTGAA
	CXXC1_1	60	40	ChIP	-518 to -390	GAGGAGTAAAAGCCGGAAAGAGT	AGTCTCTTTCCCCTTCCACATGT
	CXXC1_2	60	40	ChIP	-420 to -313	CATTAATACATGTGGAAGGGGAAA	TCAGGCAGAGAGGTTCACAGGTA
	CXXC1_3	60	40	ChIP	-218 to -104	AATTCGACCCACGTACGGAAT	CGTACGGCGGCTGCTTG
	SMAD4_1	60	40	ChIP	-520 to -425	GGCTGCCAGAAAGAGAAGGAA	AGTCGACAGCTCTGGGGAGAA
	SMAD4_2	60	40	ChIP	-468 to -312	GAACTCCTACAACCTAGCCACCTT	GAGTGGGCGTCCAGTAAGTGTT
	SMAD4_3	60	40	ChIP	-206 to -48	CCAGGCCCAGGTCCAGATT	TCCGGGTAATTTCAGGGTTTG
	DCC_1	60	40	ChIP	-597 to -497	GAAGAGAGAACACAGACGACATGAGA	ACACACAGCGGACAGGACAAAG
	DCC_2	60	40	ChIP	-469 to -372	GAAGGGAGTTGGATCAGGTGATT	GGAGGTGGCCAGGATGTTTAC
	DCC_3	60	40	ChIP	-308 to -186	CAAACACGGTCGCTGCAAA	TTGCCTGGTCTGCGTATCAAT
	DCC_4	60	40	ChIP	-153 to -46	TTTTTCCCTAAGCCATAGTCTGTCT	CGTACTACACATTGATCCGGAGAA
	DCC_5	60	40	ChIP	-63 to +43	CCCTTCCAGCCCTCCACTT	GGGGTGGAGCGCTCTCACT
	DCC_6	60	40	ChIP	-6 to +110	GCGGACACCCCAGTAACAAGT	TGGAGATGCTGTTGGTTTTTCTT
	DCC_7	60	40	ChIP	-78 to +174	GGCTCTCGGAAGAAAAACCAA	GCATTAAAAGGTTGAGGCTGGT
	DCC_8	60	40	ChIP	-222 to +374	CTCCTGGATGTGGTTTATTGATGA	GCAGGCAGCAAAAAGTTCCA
	DCC_9	60	40	ChIP	-228 to +467	GGGCTCGGGATCTCTTGGA	TCGTCCTCTTCCTCCACCTCTT
	MBD2_1	60	40	ChIP	-548 to -434	TAGGCTGGACTTTCAACATAATGG	TAGAATGACCAGAATCCCTAAATCC
	MBD2_2	60	40	ChIP	-359 to -264	AAGGTTCCAGGAGAATCTGTCAAT	ATAATGAAGGAATGAAGCAAAGTGTC
	MBD2_3	60	40	ChIP	-224 to -114	GGCTATCACCCCCTTAAATGAAT	TCCACCATTATGTGATTTTACCTATGT